The Cellular Location of the *Prevotella ruminicola* β 1,4-D-Endoglucanase and Its Occurrence in Other Strains of Ruminal Bacteria

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Introduction

Ruminant animals have developed the capacity to digest cellulose by exploiting a symbiotic relationship with cellulolytic ruminal bacteria. Enumeration studies have indicated that non-cellulolytic bacteria outnumbered the cellulolytics even when wheat straw was the only ingredient in the diet, and later work indicated that there was a crossfeeding of "cellulose fragments" from cellulolytic to non-cellulolytic bacteria. *Prevotella ruminicola* is usually described as a starch-degrading bacterium, but it is able to utilize water soluble cellodextrins, and some strains have considerable carboxymethylcellulase (CMCase) activity.

The CMCase of *Prevotella ruminicola* $B_1$4 degrades CMC at a rapid rate, but it lacks a cellulose-binding domain and cannot degrade crystalline, acid-swollen or ball-milled cellulose at a significant rate. The addition of a cellulose-binding domain to the C-terminus of the CMCase increased the rate of native cellulose digestion 10-fold, and this finding has been used as a basis for creating a cellulolytic bacterium that can digest cellulose at low pH.

Materials and Methods

P. ruminicola strains TC1-1, TF1-3 and TS1-5 were provided by H. J. Flint. *P. ruminicola* strains 23, 118B, 20-78, 20-63, M384, GA-33, 2202 and D42f were provided by M. Cotta. *B. ovatus*, *B. distasonis*, *R. amylophilus*, and *S. ruminantium* were provided by T. Miller. The bacteria were grown anaerobically in a semi-defined medium.

Cells were harvested by centrifugation. The cell suspension was sonicated, and cell debris was removed by centrifugation. The whole cells, cell-free supernatants and cell-extracts were assayed for CMCase, mannanase and xylanase activities. Samples were incubated at 55°C for 20 min and loaded onto 8% SDS polyacrylamide gels containing 2% polysaccharide. Western immunoblotting was performed using antisera prepared against the 40.5kDa cloned CMCase purified from *E. coli*. Antiserum was reacted with whole cells to insure that it did not contain antibodies against other *P. ruminicola* proteins.

The CMCase was purified via a scheme employing ammonium sulfate, phenyl sepharose, Hypatite C and Q-sepharose. The CMCase-containing fractions were concentrated using a 30,000 MW cutoff filter.

Results and Discussion

Prevotella ruminicola $B_1$4, TC1-1, TF1-3 and TS1-5 all produced immunologically crossreactive 88 kDa and 82 kDa CMCases. *P. rumincola* 23, 118B, 20-63 and 20-78 had much lower CMCase activities and Western blots showed no cross-reaction with the $B_1$4 CMCase antiserum. *Fibrobacter succinogenes* S85 and *Selenomonas ruminantium* HD4 and D produced CMCases, but these enzymes were smaller and did not cross-react with the $B_1$4 CMCase antiserum. The $B_1$4 CMCase antiserum inhibited the $B_1$4, TC1-1, TF1-3 and TS1-5 CMCase activities and agglutinated these cells, but it had no effect on the other strains or species. Based on these results, the $B_1$4 CMCase is a strain specific enzyme that is located on the outside surface of the cells. *P. ruminicola* $B_1$4 cultures grown on sucrose did not have significant CMCase activity, but these cells could bind purified 88 and 82 kDa CMCase, but not 40.5 kDa CMCase. Because the 40.5 kDa CMCase is a fully active, truncated form of the CMCase, it appears that the N-terminal domain of the 88 kDa $B_1$4 CMCase anchors the CMCase to the cells. Cells grown on cellobiose produced at least 10-fold more CMCase than the sucrose-grown cells, and the cellobiose-grown cells could only bind 15% as much CMCase as sucrose-grown cells. Virtually all of the CMCase activity of exponentially growing cultures was cell-associated, but CMCase activity was eventually detected in the culture supernatant. Based on the observation that the 88 kDa CMCase was gradually converted to the 82 kDa CMCase when cultures reached stationary phase without a change in specific activity, it appears that the 82 kDa protein is probably a proteolytic degradation product of the 88 kDa CMCase.

The CMCase hydrolyzed carboxymethylcellulose and barley glucan but not xylan or mannan. The activity varied 20-fold when *P. ruminicola* $B_1$4 was grown on different sugars. The highest activities were observed with mannose, cellobiose or xylose and little activity was observed with sucrose, arabinose or rhamnose. Because cells that were grown with sucrose in addition to mannose or sucrose in addition to cellobiose had at least 10-fold less activity than cells grown on mannose or cellobiose, respectively, it appeared that sucrose acts as a repressor. Arabinose also decreased activity but not to as great an extent as sucrose. The complexity of endoglucanase expression was also illustrated by the observation that the combination of two inducers (mannose and cellobiose) gave at least 5-fold less activity than a single inducer (cellobiose or mannose). *P. ruminicola* $B_1$4 also had significant xylanase and mannanase activities, but these activities were present in proteins that had lower molecular weights than the endoglucanase, and these proteins did not cross react with antibody made against the endoglucanase. Mannanase activity has a similar pattern of expression to the endoglucanase, while the xylanase was not induced or repressed by the same sugars or combinations of sugars. The xylanase activity was greatest when xylan was the energy source for growth, but xylose was a very poor inducer of xylanase activity.

Conclusions

Genetic modification of the *P. ruminicola* $B_1$4 CMCase is still a reasonable mechanism for creating an acid-resistant cellulolytic ruminal bacterium.